

Effect of bisphosphonates on nitric oxide production by inflammatory activated chondrocytes

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Abstract

Objective

Bisphosphonates have been reported to possess anti-inflammatory and cartilage protective effects in animal arthritis models but not much is known about their direct effect on chondrocytes. In this study we evaluate the effect of bisphosphonates on nitric oxide (NO) production by activated chondrocytes.

Methods

Isolated bovine chondrocytes and bovine cartilage explants were used. In the second part of the study human cartilage explants (osteoarthritis (OA) and non-OA cartilage) were used. The isolated chondrocytes and cartilage explants were pre-incubated with clodronate, pamidronate or risedronate and stimulated with IL-1 and TNF- α (10 ng/mL, 48 h). NO production was quantified using the Griess assay.

Results

In bovine cultures, clodronate (10^{-4} mol/L) and pamidronate (10^{-6} mol/L) showed a small inhibition of NO production (up to 15 % and 25% respectively), whereas risedronate had no effect. In the human cartilage cultures no effect of BPs on the NO production was detected except for the highest concentration of clodronate tested (10^{-4} mol/L) which demonstrated a small enhancement (19%) in NO production reaching significance in the non-OA group.

Conclusion

BPs have a modest effect on NO production by inflammatory activated chondrocytes only in the higher concentrations, indicating that the clinical relevance of these effects is probably negligible.

Key words

Chondrocytes, bisphosphonates, nitric oxide.

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Received on August 4, 2006; accepted in revised form on February 1, 2007.

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Introduction

Destruction of articular cartilage ultimately leading to joint destruction is a common event in rheumatoid arthritis (RA) and osteoarthritis (OA). Levels of nitrite, a stable end product of nitric oxide (NO), are elevated in serum and synovial fluid of both RA and OA patients (1). Chondrocytes are a major source of NO in the inflamed joint (2). Different catabolic effects of NO are described on chondrocytes including inhibition of matrix synthesis (3, 4), activation of metalloproteinases (5, 6), promotion of oxidant injury (7) and induction of apoptosis (8).

Oral glucocorticoids are widely used as anti-inflammatory treatment for patients with RA. However use of these drugs is considered as a risk factor to develop osteoporosis in those RA patients (9, 10). Bisphosphonates (BPs) are strong inhibitors of osteoclast mediated bone resorption and are useful in the prevention and treatment of glucocorticoid induced osteoporosis in patients with RA (11-13). Based upon their structure, BPs can be classified into nitrogen-containing BPs and non-nitrogen-containing BPs. Non-nitrogen-containing BPs, such as clodronate, can be metabolized to a cytotoxic analogue of adenosine triphosphate (ATP) leading to apoptosis of the osteoclast (14). Nitrogen-containing BPs (*e.g.* risedronate and pamidronate) inhibit the mevalonate pathway reducing the prenylation of small GTP-binding proteins which are essential for normal osteoclast function and survival (15, 16). It has been reported that nitrogen-containing BPs have anti-apoptotic effects on osteocytes and osteoblasts by mechanisms independent of the mevalonate pathway (17, 18). Moreover, data indicate anti-inflammatory and chondroprotective properties of BPs in animal models of RA (19-21). Currently, little is known about the direct effects of bisphosphonates on chondrocytes. In previous *in vitro* studies we have shown that BPs are not toxic for articular chondrocytes (22) and that BPs possess antioxidant properties by inhibiting chondrocyte lipid peroxidation (23). The aim of the present study is to evaluate the influence of BPs on NO production by inflammatory activated chondrocytes.

Materials and methods

Culture of bovine isolated chondrocytes

Bovine articular chondrocytes were obtained from the tarsometatarsal joints of 2 to 4 year-old animals, immediately after slaughter. Chondrocytes were isolated using hyaluronidase (Roche, Germany), protease (Sigma, St-Louis, USA) and collagenase type 1A (Sigma) as described before (24). Primary isolated chondrocytes were cultured in a monolayer at 10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin (Invitrogen), enriched with CaCl_2 (final concentration 1.6 mmol/L) and 10% foetal bovine serum (FBS, Sigma) (DMEM +10% FBS) for 24 h (22).

Culture of cartilage explants

1) *Bovine cartilage explants.* Explants of bovine articular cartilage were aseptically dissected from the tarsometatarsal joints of 2 to 4 year-old animals. Full-thickness cartilage explants were obtained using a 4 mm biopsy punch (PFM, Köln, Germany). The explants were pooled and cultured in calcium enriched DMEM +10% FBS (25).

2) *Human cartilage explants.* The human cartilage explant cultures were divided into 2 groups. The first group ($n = 10$) contained cartilage from patients undergoing joint replacement for destructive osteoarthritis (OA). The second group ($n = 13$) contained macroscopically normal cartilage obtained from organ donors ($n = 3$), from autopsy ($n = 2$) or from patients undergoing joint replacement for hip fracture ($n = 8$) (non-OA). Cartilage obtained from patients with rheumatoid arthritis, active infection, malignancy and patients treated with statins, bisphosphonates or glucocorticoids were excluded. The median age of the OA group was 62 years (range 27-78) and of the non-OA was 64 (range 34-83). Full thickness cartilage explants were obtained and cultured identically as the bovine explants.

Viability, assessed by calcein AM (Molecular Probes, Invitrogen)/ propidium iodide (PI, Sigma) staining of the cells/ cartilage (bovine and human) explants

Competing interests: none declared.

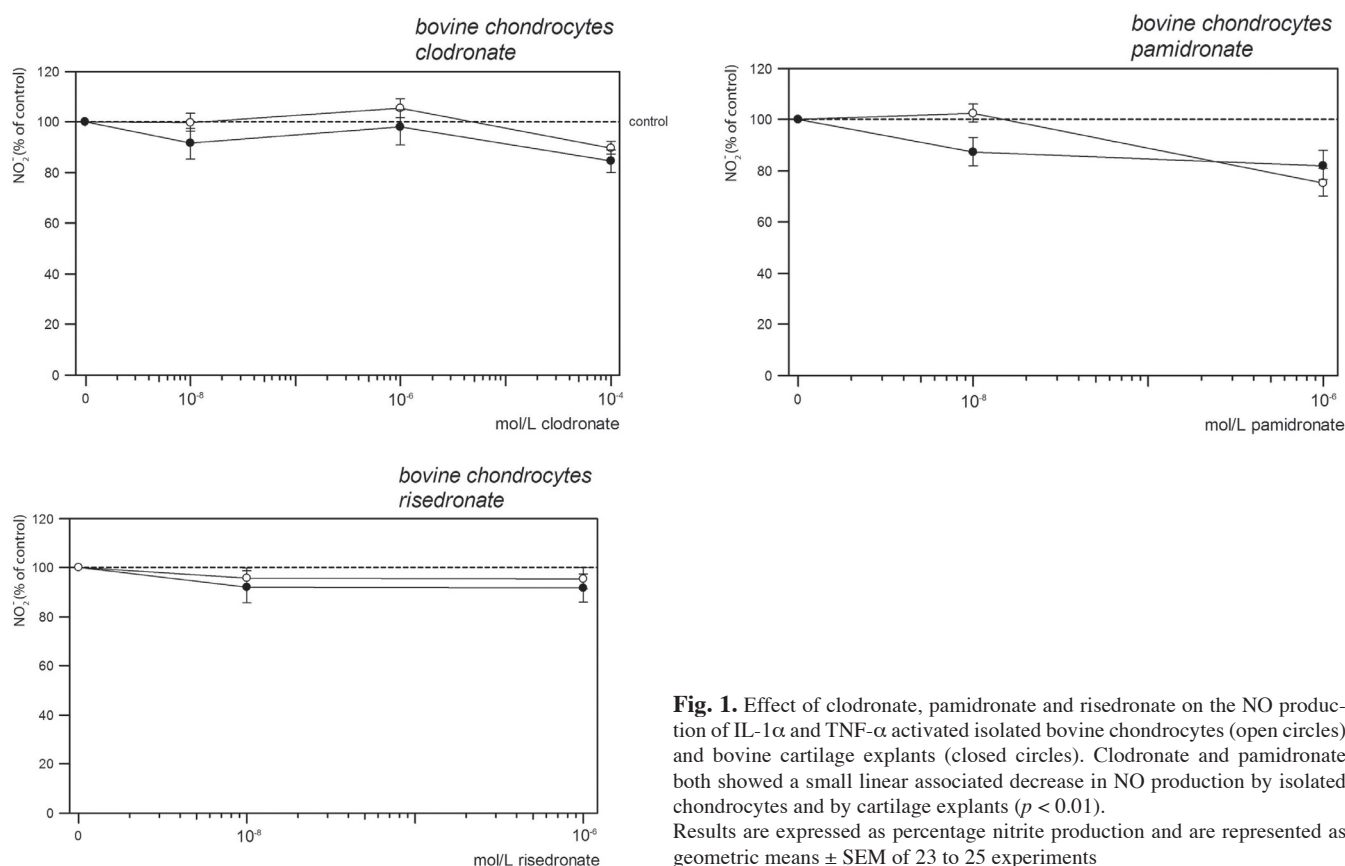


Fig. 1. Effect of clodronate, pamidronate and risedronate on the NO production of IL-1 α and TNF- α activated isolated bovine chondrocytes (open circles) and bovine cartilage explants (closed circles). Clodronate and pamidronate both showed a small linear associated decrease in NO production by isolated chondrocytes and by cartilage explants ($p < 0.01$). Results are expressed as percentage nitrite production and are represented as geometric means $\pm$ SEM of 23 to 25 experiments

always exceeded 80% at the start of the experiments (22, 25).

Preincubation with bisphosphonates

Isolated chondrocytes or cartilage explants were incubated for 48 h in medium with different concentrations of clodronate (10^{-8} , 10^{-6} , 10^{-4} mol/L) (Bonafos, Schering, Diegem, Belgium), pamidronate (10^{-8} , 10^{-6} mol/L) (Aredia, Novartis Pharma, Vilvoorde, Belgium), risedronate (powder, kindly provided by Procter & Gamble Pharmaceuticals, Cincinnati, USA) (10^{-8} , 10^{-6} mol/L) or in DMEM + 10 % FBS alone as a control. Pre-incubation period and concentrations of BPs were determined in previous work by our group (22, 25).

Stimulation of isolated chondrocytes and cartilage explants

The medium was replaced with calcium enriched DMEM without FBS and with or without the different BPs. The cultures were stimulated with human interleukin-1 (IL-1 α or IL-1 β , PeproTech House, London, UK) and human tumor necrosis factor α (TNF- α , PeproTech

House) each in a concentration of 10 ng/mL for 48 h. Both, the stimulation period and concentration of IL-1/TNF- α were chosen based on previous studies (24, 26). IL-1 α and IL-1 β was used for bovine and human cartilage, respectively (24, 27). The arginine analogue, L-N^G-monomethyl arginine (L-NMMA, Sigma, 10^{-4} mol/L) was used as a positive control and inhibited the NO production $\geq 70\%$. After culture, supernatants were collected and stored at -20°C for further analysis.

NO assay

NO production was detected by measuring the stable NO metabolite, nitrite, using a spectrophotometric method based on the Griess reaction (26). Results were expressed in $\mu\text{mol/L}$ nitrite per well for the chondrocyte cultures. The cartilage explants were weighed and NO production was expressed as $\mu\text{mol/mg}$ nitrite. The detection limit was $0.01 \mu\text{mol/L}$. The intratest variability of the NO production by isolated chondrocytes was lower than for the cartilage explants (6% compared to 20%).

Statistics

All statistical analyses were performed using SPSS 12.0. Data were logarithmically transformed in order to obtain normal distribution. Repeated measures ANOVA and independent sample t -test were used where appropriate. A p value < 0.05 was considered significant.

Results

Effect of BPs on NO production of bovine isolated chondrocytes and cartilage explants

No spontaneous NO production was found by unstimulated isolated bovine chondrocytes or by cartilage explants. Stimulation of the isolated chondrocytes and explants with IL-1 α and TNF- α resulted in enhanced NO production (median $21.09 \mu\text{mol/L}$, range 6.49 to $54.86 \mu\text{mol/L}$ for the isolated chondrocytes and median $1.08 \mu\text{mol/mg}$, range 0.34 - $1.95 \mu\text{mol/mg}$ for the cartilage explants). Clodronate and pamidronate both showed a small linear associated decrease in NO production, primarily due to the highest concentrations ($p < 0.01$) where clodronate (10^{-4}

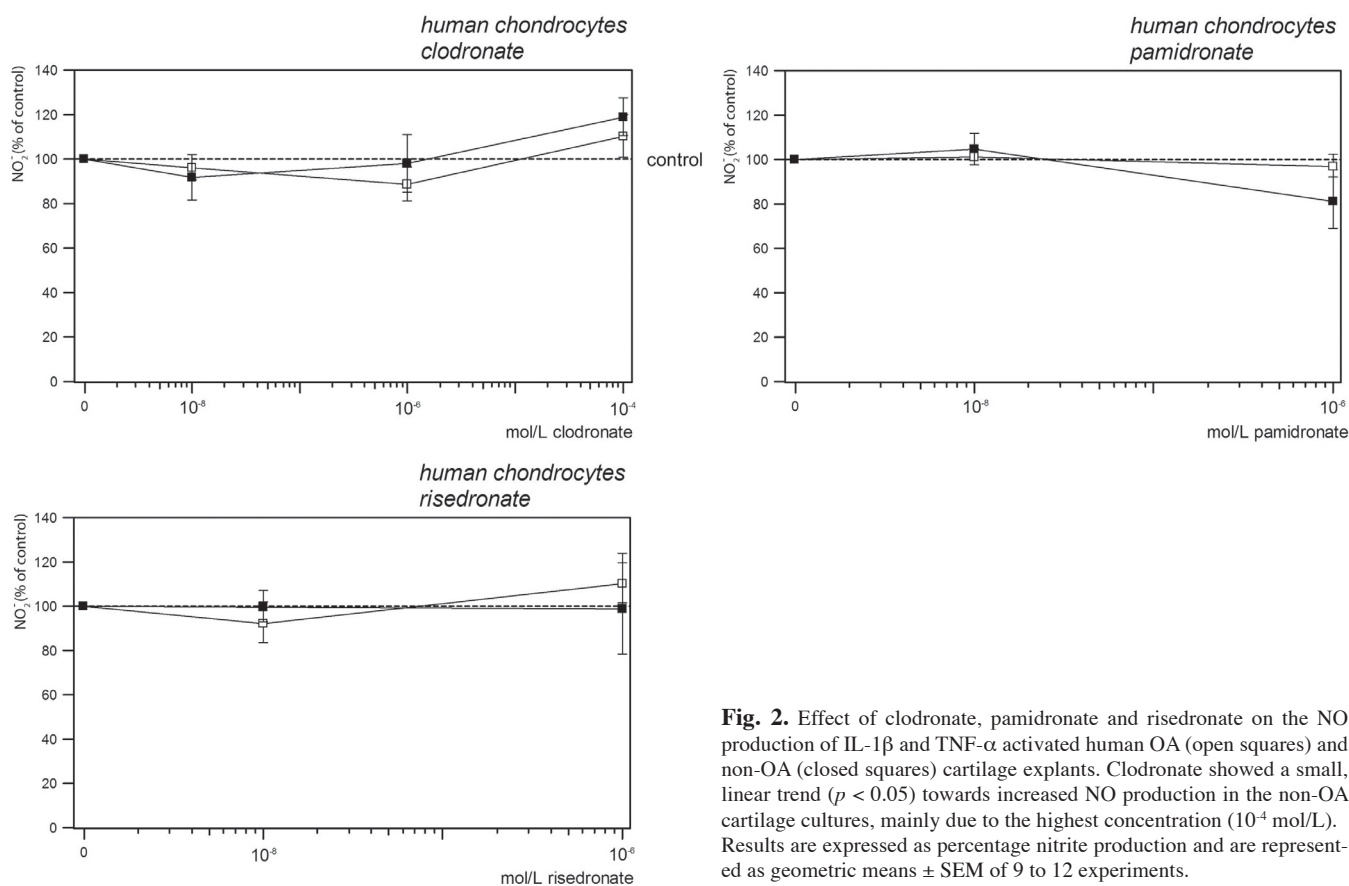


Fig. 2. Effect of clodronate, pamidronate and risedronate on the NO production of IL-1 β and TNF- α activated human OA (open squares) and non-OA (closed squares) cartilage explants. Clodronate showed a small, linear trend ($p < 0.05$) towards increased NO production in the non-OA cartilage cultures, mainly due to the highest concentration (10^{-4} mol/L). Results are expressed as percentage nitrite production and are represented as geometric means $\pm$ SEM of 9 to 12 experiments.

mol/L) and pamidronate (10^{-6} mol/L) reduced the NO production in isolated chondrocytes by 10 % and 25% respectively, and by 15% and 18% in the cartilage explants (Fig. 1). Risedronate had no effect on the NO production of bovine isolated chondrocytes and cartilage explants ($p \geq 0.2$).

Effect of BPs on NO production of human cartilage explants

In a second series of experiments we investigated the effect of BPs on human cartilage explants. The human cartilage explant cultures could be divided into two groups, the OA and non-OA group. No basal NO production was found in both groups. In the non-OA group one sample had to be excluded due to low NO production after stimulation. IL-1 β and TNF- α stimulation resulted in enhanced NO production in both non-OA cartilage (median 1.36 $\mu\text{mol}/\text{mg}$, range 0.45 – 2.94 $\mu\text{mol}/\text{mg}$) and OA cartilage (median 2.07 $\mu\text{mol}/\text{mg}$, range 0.94 – 2.53 $\mu\text{mol}/\text{mg}$) and there was no difference in NO production between OA and non-OA cartilage ($p = 0.2$).

No effect of BPs on NO production of human cartilage explants could be detected ($p > 0.2$), except for clodronate which showed a small, linear trend ($p < 0.05$) towards increased NO production in the non-OA cartilage cultures. As shown in Figure 2, this was predominantly due to the highest concentration (10^{-4} mol/L) of clodronate tested which enhanced the NO production by 19%.

Discussion

Data indicate that BPs have anti-inflammatory and chondroprotective effects in animal models of RA (19–21). In RA and OA, chondrocytes are indicated as the major source of NO (2). However, little is known about the effect of BPs on the NO production of cells, particularly in chondrocytes. In this study, in both bovine isolated chondrocytes as well as in bovine cartilage explants, a small inhibition, primarily in the highest concentrations tested, was found for clodronate and pamidronate, while risedronate had no effect. Bovine chondrocytes were used since they are readily available and since they show a similar

profile of susceptibility to inhibition of NO production by different anti-inflammatory drugs as human chondrocytes (28). Furthermore, cartilage explants were also studied because it is known that the extracellular matrix is important for chondrocyte metabolism (29). However, since species differences in NO production have been described (27) we also evaluated the effect of BPs on human cartilage explants (OA and non-OA). No reduction in NO production of human cartilage was found for pamidronate and risedronate. Clodronate showed a small increase, predominantly caused by the highest concentration, which reaches significance in the non-OA group. The subtle differences in effects of BPs on NO production between the bovine and human cultures might be related to species differences. Distinct pathways of iNOS induction and regulation in bovine and human chondrocytes have been described, where bovine chondrocytes use p38 mitogen-activated protein and protein tyrosine kinase and human chondrocytes most likely only protein tyrosine kinase (27). In addition,

the layer of human cartilage obtained is thicker than that of the bovine cartilage and this implicates that compounds need to diffuse deeper through the matrix to reach the cells. The age of cartilage is also diverse (bovine cultures are from young animals, while the human cultures are mostly from older persons). Additionally, one should also keep in mind that each bisphosphonate has its own physicochemical and biological characteristics and therefore it is impossible to extrapolate data from one compound to another, neither from one cell type to another (30). In literature, inhibition in NO production by clodronate in stimulated macrophage-like and microglia cells have been described (31, 32). The nitrogen-containing BPs, alendronate and ibandronate had no effect on the NO production in macrophage-like cells (31, 33) and the reduction in NO production by pamidronate was most likely due to cytotoxic effects. However, the concentrations of BPs used in our study were previously reported to have no influence on the viability of chondrocytes (22).

In contrast to other reports, we did not find spontaneous NO release in the osteoarthritis affected cartilage explants (28, 29, 34). This discrepancy might be explained by some differences in experimental protocol. First of all, the human cartilage explants were pre-incubated during 48 h and the medium was replaced before the stimulation period, meaning that if NO was produced during the first 48 h of culture, this was not measured in our assay. Secondly, during the stimulation period we omitted FBS to exclude aspecific stimulatory effects. Indeed in preliminary experiments we measured a spontaneous NO production in the presence of FBS in bovine cartilage explants which was not found in the absence of FBS (data not shown). Thirdly, we did not use cartilage of the osteoarthritic lesions, which might also explain the fact that no higher stimulated NO production in the OA group compared to the non-OA group was measured.

Taken together, the effects found of the BPs on NO production by activated chondrocytes were modest and only present at higher concentrations, indicating that the clinical relevance of these effects is probably negligible.

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